WEST Search History

DATE: Wednesday, July 10, 2002

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	DB=USPT,JPAB,EPAB,DWPI; PLUR=YES; OP=ADJ		
L18	l11 and (serum)	7	L18
L17	l11 and (aprotic or aceton$6 or sulfoxide or formamide or dimethyl)	5	L17
L16	aprotic same cell$4	779	L16
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L14	cell$4 same (assay$ or immunoassay$)	56121	L14
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L12	L7 and (saponin and methanol)	20	L12
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L10	L9 and (saponin same methanol)	0	L10
L9	L5 and (assay$ or immunoassay$)	220	L9
L8	L5 and (saponin same methanol)	1	L8
L7	L6 and (assay$ or immunoassay$)	642	L7
L6	l1 and l2	764	L6
L5	L4 or l3	266	L5
L4	(cell$4 same penetra$5) same (cytomet$2)	48	L4
L3	(cell$4 same permeab$9) same (cytomet$2)	225	L3
L2	(cell$4 same fix$5) same (cytomet$2)	764	L2
L1	((cell$) same (fix$)) same (cytomet$)	870	L1

END OF SEARCH HISTORY

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L11: Entry 2 of 7 File: USPT Jun 19, 2001

DOCUMENT-IDENTIFIER: US 6248514 B1
TITLE: Methods for measuring viral infectivity

Brief Summary Paragraph Right (2):
Traditionally, infectivity particles are measured in culture by a plaque-forming unit assay (pfu) that scores the number of viral plaques as a function of dilution. An alternative to the pfu assay is the tissue culture infective dose procedure (TCID.sub.50), which estimates infectivity as a function of intracellular staining for an antigen by direct immunofluorescence. The methods suffer from limitations including a high degree of inter-assay variability and are affected by such factors as virus replication status, vector characteristics, and virus-cell interactions.

Brief Summary Paragraph Right (3):
More recently, flow cytometry or FACS (fluorescence-activated cell sorter) assays have been used to measure the number of infected cells in cell cultures infected at relatively high multiplicities of infection. For example, Saalmuller and Mettenleiter (J. Virol. Methods 44:99-108 (1993)) disclose the identification and quantitation of cells infected by recombinant pseudorabies virus mutants by the reaction of intracellular .beta.-galactosidase expressed during infection with recombinant viruses with a fluorogenic substrate, followed by detection of positive cells in flow cytometry. Morris et al. (Virology 197(1):339-48 (1993)) studied the process of productive and non-productive recombinant AcMNPV infection in cultured cells by immunostaining cells to detect the reporter CAT gene product.

Detailed Description Paragraph Right (1):
The present invention provides a novel flow cytometry-based analytical method that can detect the intracellular presence of adenovirus protein(s). The method is rapid (48 hours), sensitive, accurate and precise (<40% CV), rugged and broadly applicable. In one aspect of the invention, important variables are identified that affect measurement of infectious virions. These variables are both physical (initial virus particle concentration, time for adsorption of the virus to cells, length of the assay, and initial cell density) and biological (type of vector, the therapeutic transgene, and the target cell) in nature.

Detailed Description Paragraph Right (2):
The assay methods provided by the invention are useful for enhancing the understanding of the underlying mechanisms of adenoviral infection, which will lead to improved vector delivery methodology. The methods are also useful for addressing issues fundamental to gene therapy, and can be used to support product development. The assay has allows reduced cycle time for bulk product and formulated material release as well as provides a method for ongoing studies with product formulation and stability. The assay methods of the invention can also be used for analysis of clinical samples for infectious adenovirus in body fluids such as serum or urine. In addition, the methods of the invention can be used to quantify neutralizing antibody titers without many of the constraints of assays in current use.

Detailed Description Paragraph Right (3):
Yet another utility of the assay methods of the invention is that the methods can detect replication of replication-competent virus particles (such as, for example, adenovirus) in cell targets that do not support replication of replication deficient recombinant viral vectors (e.g., E1-deleted recombinant adenovirus). For example, one can use the assays as impurity assays for replication competent adenoviral vectors (RCA). In addition, the ability of different forms of RCA to replicate in

specific cell targets can be screened using the methods of the invention.

Detailed Description Paragraph Right (9):
The assays involve infecting a cell line with a preparation of a viral vector particles. For example, the 293 cell line is suitable for amplification of replication-deficient recombinant adenovirus (rAd) constructs.

Detailed Description Paragraph Right (11):
Antibodies used for detection can be polyclonal, monoclonal, or include mixtures of such antibodies. Typically, the detection is done directly by using a fluorescein-conjugated antibody directed against the viral polypeptide. However, indirect assays are also possible,in which the antibody directed against the viral polypeptide is then reacted with a fluorescein-labeled antibody. Any fluorescent label compatible with flow cytometry can be used.

Detailed Description Paragraph Right (12):
In some embodiments, the assays of the invention include determining the total number of virus particles in a viral preparation. This can be measured by any of a number of traditional techniques. For example, an aliquot of a virus preparation can be prepared in a buffer containing 0.1% sodium dodecyl sulfate (SDS), after which the optical absorbance is measured at 260 nm (Maizel et al. Virology 36:115-125 (1968)). Total particle counts can also be obtained by preparing a sample of the viral preparation for electron microscopy, and simply counting the number of particles. A further technique for particle enumeration can include the use of anion-exchange chromatography (Huyghe et al. (1995) Human Gene Therapy 6:1403-1416).

Detailed Description Paragraph Right (22):
Total particle number was obtained by the "SDS/OD.sub.260 " method and anion exchange chromatography methods described above. In both assays the measured total particle concentration was 1.0.times.10.sup.12 /ml.

Detailed Description Paragraph Right (23):
Infectious particles were titered by TCID.sub.50 assay as described by Huyghe et al. (Human Gene Therapy 6:1403-1416 (1995)). In brief, 293 cells were plated into a 96-well microtiter plate: 100 .mu.l of 5.times.10.sup.5 cells/ml for each well in complete MEM (10% bovine calf serum; 1% glutamine) media (GIBCO BRL). In a separate plate, a 2501-.mu.l aliquot of virus sample diluted 1:10.sup.6 was added to the first column and was serially diluted two-fold across the plate. Seven rows were used for samples. One row was used for a negative control. A 100-.mu.l aliquot of each well was transferred to its identical position in the 293 seeded plate and allowed to incubate a 37.degree. C. in a humidified air/7% CO.sub.2 incubator for 2 days. The media was then decanted by inversion and the cells fixed with 50% acetone/50% methanol. After washing with PBS, the fixed cells were incubated for 45 minutes with a FITC-labeled anti-Ad5 antibody (Chemicon International #5016) prepared according to the kit instructions. After washing with PBS, the plate was examined under a fluorescent microscope (490 mm excitation, 520 mm emission) and scored for the presence of label.

Detailed Description Paragraph Right (25):
The low ratio assay was performed as follows. 1.times.10.sup.6 293 cells (human embryonic kidney cells, ATCC CRL 1573) were seeded per well on 4 6-well dishes. The final volume per well was 1 ml. After about 2 hr, the medium (Dulbecco's modified Eagle's medium (DME high glucose) containing 4500 mg/ml D-glucose, supplemented with 5% defined, iron-supplemented bovine calf serum, 2 mM L-glutamine, and 1 mM sodium pyruvate) in each well was aspirated and replaced with 1.1 ml of medium (without serum) containing diluted virus. Adsorption was allowed to occur for 60 minutes, after which an additional 2 ml of virus-free medium was added to each well. After about 42 hr, the infected cells cultures were processed for flow cytometry analysis.

Detailed Description Paragraph Right (27):
The results are shown in Table 1. By the traditional TCID.sub.50 assay, the total particle number to infectious unit ratio was 63:1. As is evident in the table, as the total particle number to cell number ratio decreased, the calculated total

particle number: infectious unit ratio also decreased to as low as 12:1, thereby providing a value for infectious titer that was about 5-fold higher than the traditional assay. Thus, this low ratio assay provides an unexpectedly better (i.e. much more accurate) enumeration of the number of infectious particles in a viral preparation than traditional methods for titration. The consequences of such accurate measurements proved by the instant invention are especially important in calculating the effective doses of recombinant viruses for therapeutic use.

Detailed Description Paragraph Right (28):
This Example describes a study directed to identifying and optimizing those parameters critical to the measurement of infectious viral particles and to ensuring that the assay is generally applicable to all types of adenovirus preparations. The results indicate that the most important variables affecting the measurement of infectious virions are initial particle concentration, time for adsorption of the virus, and length of the assay. The results also demonstrate that one can determine virus infectivity by measuring the fraction of cells in a sample population that express a vector-associated gene. This is significant because it enables one to assess infectivity of recombinant adenovirus preparations on targets without using reporter constructs, and allows one to directly compare the transduction efficiency of different gene therapy modalities.

Detailed Description Paragraph Right (36):
293 cells were seeded at a density of 1.6.times.10.sup.5 cells/cm.sup.2 in 96-well flat-bottom microtiter dishes using 100 .mu.L of 293 cell media. Dilutions of adenovirus samples were prepared in separate 96-well plates and added to the cells to bring the final volume in each well to 200 .mu.L. Plates containing cells with virus were subsequently incubated in a humidified air/7% CO.sub.2 incubator at 37.degree. C. for 2 days. Seven rows were used for samples while the eighth row was used as a negative control. Media was decanted and the cells fixed briefly with a 1:1 mixture of methanol and acetone. After washing with Dulbecco's phosphate-buffered saline (D-PBS), the cells were stained for 45 minutes with a FITC-conjugated murine IgG.sub.1 anti-adenovirus monoclonal antibody (Chemicon). The cells were examined by microscopy using epifluorescence illumination (490 nm excitation, 520 nm emission) and scored (i.e., + or -) for the presence of virus. Infectious titer was calculated using a modification of the TiterPrint Analysis program (Lynn (1992) BioTechniques 12: 880-881).

Detailed Description Paragraph Right (38):
293 cells were seeded at a density of 1.6.times.10.sup.5 cells/cm.sup.2 in 6-well dishes in 1.0 ml media. The reasons for this high density of cells were (i) to generate a confluent monolayer to ensure that virus diffusing to the bottom of the well would probably adsorb to a cell, and (ii) to minimize changes in cell proliferation during the course of the assay. Each plate was incubated overnight in a humidified air/7% CO.sub.2 incubator at 37.degree. C. to facilitate 293 cell attachment. For the virus adsorption step, growth media was gently removed from each well followed by the addition of 100 .mu.L of the diluted virus sample and 400 .mu.L of 293 cell media. Each plate was returned to the CO.sub.2 incubator for an additional 3 hours. An additional 2 ml of 293 cell media was added and all plates were incubated for 48 hours (unless otherwise indicated). Forty-eight hours was chosen because it was the longest time for hexon expression but prior to the completion of a full viral replication cycle. To stain the cells for adenovirus capsid proteins, media was removed and the cells harvested using Trypsin/EDTA. Cells were washed with D-PBS using gentle centrifugation (120.times.g), resuspended in 300 .mu.l of cold D-PBS. Cells were fixed and permeabilized for 10 minutes with cold 1:1 (v/v) methanol:acetone. Again cells were washed and resuspended in 50 .mu.l of D-PBS with 1% (v/v) bovine calf serum. Cells were stained for 45 minutes at 37.degree. C. with either various FITC-conjugated murine IgG.sub.1 anti-adenovirus monoclonal antibody preparations, a FITC-conjugated goat anti-adenovirus polyclonal antibody preparation, or appropriate FITC-conjugated isotype binding controls. Using either a FACScan.TM. or FACSCalibur.TM. flow cytometer (Becton Dickinson), between 10000 and 50000 events were acquired in list-mode format for forward scatter (FSC), side scatter (SSC), and FITC fluorescence (FL-1) parameters. Data were analyzed using Cell Quest.TM. cytometry software (Becton Dickinson). The percentage of adenovirus positive cells in each sample was used to determine the infectious titer.

Detailed Description Paragraph Right (40):
SaOS-2 cells were seeded at a density of 1.0.times.10.sup.5 cells/cm.sup.2 in 6-well dishes in 1.0 mL of SaOS-2 cell media. They were then infected, cultured, fixed and stained as described in the preceding section with the following exceptions. For detection of the p110RB transgene product, cells were fixed and permeabilized for 10 minutes with 3 ml of cold (-20.degree. C.) 70% (v/v) ethanol, not stained for adenovirus hexon, but rather were stained using a FITC-conjugated anti-p110RB murine monoclonal antibody, 3C8 (Wen et al. (1994) J. Immunol. Meth. 169: 231-240). A FITC-conjugate IgG.sub.2a isotype control was used to adjust for non-specific binding in p110RB experiments. For detection ofp53 expression, cells were fixed as described for the infectivity assay, not stained for adenovirus hexon, but stained using a PE-labeled anti-p53 murine monoclonal antibody (PharMingen). A PE-labeled isotype control was used to adjust for non-specific binding in p53 experiments. Using either a FACScan.TM. or FACSCalibur.TM. flow cytometer (Becton Dickinson), 20000 events were acquired in list-mode format for forward scatter (FSC), side scatter (SSC), FITC fluorescence (FL-1), or Phycoerythrin (FL-2) parameters. Data were analyzed using Cell Quest.TM. cytometry software (Becton Dickinson). The percentage of p110RB or p53 positive cells in each sample was assessed in a manner similar to what was used for hexon expression in 293 infected cells.

Detailed Description Paragraph Right (43):
In initial studies, we determined whether it was feasible to assess adenovirus infectivity by flow cytometry. We defined key parameters for development: target cells expressing adenovirus E1 proteins that complement in trans corresponding deletions within recombinant adenovirus vectors, appropriate dilutions of virus preparations, methods to fix and permeabilize cells that did not compromise the viral epitope yet maintained enough cell integrity for flow analysis, a mechanism to readily discriminate infected from uninfected cells by flow cytometry, and a means to calculate the infectious titer from such data. 293 cells were chosen because they were readily available and could support the proliferation of replication-deficient adenovirus (Graham and Prevec (1991) "Manipulation of adenovirus vectors." In Methods in Molecular Biology--Gene Transfer and Expression Protocols vol. 7, E. J. Murray Ed. (The Humana Press Inc., Clifton N.J.), pp. 109-128). An anti-adenovirus hexon mAb was chosen to detect expression of late phase capsid proteins because of prior experience with its use in Western blots and immunohistochemical staining; the working range was determined to be between 1 and 20 .mu.g/ml with an optimal concentration near 3 .mu.g/ml; the time and temperature for mAb incubation were shown to be 50 minutes and 37.degree. C., respectively. While testing various fix and permeabilization methods, which included 75% (v/v) ethanol, acetone, and a 1:1 (v/v) mixture of methanol and acetone, it was determined that flow cytometry could readily discriminate (approximately a 1 to 2 log shift in relative fluorescence intensity) uninfected and infected cells in the sample population.

Detailed Description Paragraph Right (44):
The last challenge was to devise a scheme to calculate infectious titer from raw flow cytometry output. Equations used in conjunction with enumerative techniques such as the plaque forming assay include parameters such as the number of foci, dilution factor, and inoculum volume (Issacs (1957) Advan. Virus Res. 4: 111-158; Dougherty (1964) "Animal virus titration techniques." In Techniques in Experimental Virology, R J C Harris, Ed. (Academic Press, N.Y.), pp. 169-223). Since the assay described herein is also an enumerative method like the pfu assay, we were able to define the relationships between assay-specific variables (sample dilution factor and inoculum volume) as well as flow cytometry data (the fraction of adenovirus hexon positive cells). Infectious titer is the product of the following: the total number of adenovirus infected cells in the sample population, the degree to which the adenovirus sample was diluted, and the reciprocal of the inoculum volume (Equation I). ##EQU1##

Detailed Description Paragraph Right (50):
Another potential caveat was whether the adenovirus hexon protein detected within cells after immunostaining and flow analysis was due directly to the input virus used for infection or arose from newly synthesized protein as a consequence of infection. To address this concern, we designed the following experiment: treat 293 cells with either two different dilutions of a recombinant adenovirus preparation or a mock-infected control for 3 hours, remove the inoculum and replace with fresh

media, and then assess sample populations at specified time points. The resulting time-course for adenovirus hexon expression in 293 cells infected with ACBSB34 is shown in FIGS. 2A-2C. Since hexon could not be detected in cells until at least 18 hours post infection, these data indicate that it arose from de novo synthesis of late phase proteins rather than from residual adenovirus particles used in the initial infection. Furthermore, the optimal window to use for assaying virus titer appears to be between 44 and 48 hours because it precedes a burst of CPE and subsequent re-infection events. Parallel time-course studies using ACNRB and ACN53 resulted in profiles of hexon expression similar to what was observed for ACBSB34, although the kinetics appeared somewhat delayed (FIGS. 2B and 2C).

Detailed Description Paragraph Right (54):
Prior to implementation of this assay for routine infectivity analyses, we evaluated its performance using standard validation criteria as well as to compare such results with those obtained using traditional infectivity measurements. The rationale of the study design focused on determining how reproducible and informative the resulting data were especially for distinct viral vectors with different growth characteristics.

Detailed Description Paragraph Right (55):
Previous experiments showed that the dose response, i.e. the percentage of cells expressing adenovirus hexon protein as a function of initial adenovirus particle concentration, was nonlinear (FIG. 2). The range of quantitation was between 1.times.10.sup.5 to approximately 2.times.10.sup.7 particles/ml; the limit for infectivity was as low as 300 I.U./ml. The test for precision, a measure of assay reproducibility, was used to study three parameters: analyst to analyst, plate to plate, and day to day variability. Analyst to analyst precision was about 10% while plate to plate ranged from approximately 5 to 15% (Table 3). Inter-assay variation was 39.3% using tests conducted on four separate days. The test for robustness, which was a measure of the ability of the assay to perform reliably under a variety of conditions, compared results of two analysts over four days using different reagents, dilutions, and different batches of ACN53, ACNRB, and replication competent type 5 adenovirus. Coefficients of variation up to approximately 41% were observed (Table 4) demonstrating that the assay was robust.

Detailed Description Paragraph Right (56):
A second aspect to the validation process was to correlate the results observed using the FACS-based assay described herein with the TCID.sub.50 assay for infectivity. Six different adenovirus preparations were tested in each assay using the same target cells. Although the results depicted in Table 5 show that the infectious titers determined using either assay were similar, the TCID.sub.50 results were often quite variable (i.e., greater than 200%). Further tests using a different preparation of ACBSB34 confirmed these results and indicated that the distribution of titer results was log-normal rather than a symmetric configuration. The geometric mean (N=12) was 3.04.times.10.sup.10 (.+-. one standard deviation, 2.02 .times.10.sup.10 to 4.59.times.10.sup.10) and 3.17.times.10.sup.10 (.+-. one standard deviation, 1.46.times.10.sup.10 to 6.86.times.10.sup.10) I.U./ml for the FACS-based and TCID.sub.50 assays, respectively.

Detailed Description Paragraph Right (58):
Since wild type or replication-competent adenovirus can infect and replicate in many human cell types, it was plausible to assume that this FACS-based assay could be adapted to differentiate between infected and naive normal cell populations. In order to investigate this further, we infected various cell lines with either preparations of type 5 adenovirus or an adenovirus type 5 variant known as dl309. Using conditions similar to what was previously described for the 293-based assay, the various samples were cultured for about 2 days after infection and analyzed by FACS for expression of hexon. The data, as shown in FIG. 4, indicate that it was feasible to monitor viral infectivity using this procedure and that the response appeared to be dependent upon the initial concentration of adenovirus particles. Also, there was close agreement between the results (i.e., the percentage of cells staining positive for hexon expression) for a given cell line. These data suggested that this approach could be used to address a number of issues: (i) to compare the relative infectivity of replication-competent virus in different cell types; (ii) to develop a strategy to detect replication-competent adenovirus (RCA) in batches of

replication-deficient adenovirus; and (iii) to use bivariate analyses to study coordinate expression of adenovirus hexon and specific cell surface determinants which could enable one to determine whether viral replication discriminates between subtypes of primary cells in a mixed target population.

Detailed Description Paragraph Right (62):
This Example describes the development of a novel flow-cytometry based method to measure the infectious titer of recombinant adenoviral preparations. This assay is rapid, sensitive, reproducible and can be completed within three days. It is a quantitative procedure that assesses infectivity as a function of the number of cells staining positively for expression of a viral antigen in a mixed target cell population. Experimental controls such as uninfected target cells and the use of a nonspecific IgG control conjugated with FITC helped to verify the assignment of adenovirus infected cells in the cell population. Additional studies using different anti-adenovirus antibody preparations for detecting expression of viral capsid proteins yielded equivalent infectious titers and further confirmed the rationale of this approach. Since this method was sensitive to the initial conditions used for infection as well as to events occurring as a result of the infection process, results were usually higher and more precise when compared to traditional assays for infectivity. Replication-deficient recombinant adenovirus constructs with distinct replication cycles, transgenes, and backbones all behaved reliably in this flow cytometry assay. Validation studies demonstrated that the assay was precise and robust for use in bulk product and formulated material release.

Detailed Description Paragraph Right (64):
One of the primary reasons why infectious titer assays gained general acceptance in virology was that they were the only methods which allowed investigators to "quantify" infectious particles in batches of either purified virus or in cell lysates. Data using these assays led to the development of two paradigms in adenovirus biology: that most particles present in adenovirus preparations are not infectious, and that multiplicity of infection (MOI)--the ratio of infectious units to target cells--is an appropriate unit for dosing in vitro and in vivo.

Detailed Description Paragraph Right (65):
The data presented herein strongly suggest that neither hypothesis is correct. The first tenet was based upon infectious titer data and circular reasoning. Unfortunately, infectious titer assays are often imprecise and detect only a small percentage of the particles in solution because the infection process is itself diffusion limited (Allison and Valentine, 1959a, 1959b, supra.; March et al. supra.; Nyberg-Hoffinan et al. (1997) Nature Medicine 3: 808-811).

Detailed Description Paragraph Left (3):
This provided the impetus to further develop this method into a reliable assay for characterization of recombinant adenovirus preparations.

Detailed Description Paragraph Table (1):
TABLE 1 INFECTIOUS TITER DETERMINATION: Ratio of TCID.sub.50 Virus Particle No. to Total Particle to Infectious Units to Concentration % Positive Calculated Titer Mean Calculated Titer Infectious Titer Cell Ratio Cells (Particle No./mL) Cells (IU/mL) (IU/mL) Ratio 18.9 0.300 6.5 .times. 10.sup.6 31.0 1.6 .times. 10.sup.10 1.87 .+-. 0.31 .times. 10.sup.10 53:1 18.3 .times. 10.sup.6 37.0 1.8 .times. 10.sup.10 (16.6%) 40.0 .times. 10.sup.6 43.0 2.2 .times. 10.sup.10 3.78 0.060 1.3 .times. 10.sup.6 12.0 3.0 .times. 10.sup.10 3.15 .+-. 0.21 .times. 10.sup.10 32:1 3.7 .times. 10.sup.6 13.0 3.3 .times. 10.sup.10 (6.7%) 8.0 .times. 10.sup.6 na na 0.756 0.012 0.26 .times. 10.sup.6 5.8 7.3 .times. 10.sup.10 8.67 .+-. 1.18 .times. 10.sup.10 12:1 0.74 .times. 10.sup.6 7.5 9.4 .times. 10.sup.10 (13.6%) 1.6 .times. 10.sup.6 7.4 9.3 .times. 10.sup.10 Calculated Titer (TCID.sub.50 Assay) 1.6 .times. 10.sup.10 IU/mL Particle No. Concentration 1.0 .times. 10.sup.12 PN/mL PN:IU Ratio 63:1

Detailed Description Paragraph Table (5):
TABLE 5 A Comparison of the Infectious Titers Determined by Different Assays Adenovirus Construct.sup..sctn. Flow Cytometry TCID.sub.50 ACNRB34 1.1 .times. 10.sup.10 0.8 .times. 10.sup.10 ACNRB 2.5 .times. 10.sup.10 2.1 .times. 10.sup.10 ACN34 1.1 .times. 10.sup.10 0.7 .times. 10.sup.10 ACN53 0.8 .times. 10.sup.10 1.3 .times. 10.sup.10 ACBGL 3.0 .times. 10.sup.10 3.6 .times. 10.sup.10 ACBSB34 13

.times. 10.sup.10 15 .times. 10.sup.10 .sup..sctn. Lot numbers for the preparations listed above were as follows: ACNRB34, lot C05; ACNRB, lot C01; ACN34, lot C01; ACN53, lot R32; ACBGL, lot R22; and ACBSB34 lot R01.

Other Reference Publication (4):
Eyler, Y.L. et al., "Flow cytometric detection of DNA tumor virus nuclear oncogene products in unfixed cells: saponin FACS of viral oncogene products," J. Virol. Meth. 46:23-27 (1994).

Other Reference Publication (6):
Lynn, D.E. et al., "A BASIC Computer Program for Analyzing Endpoint Assays," Biotechniques 12(6):880-881 (1992).

Other Reference Publication (10):
Saalmuller, A. et al., "Rapid identification and quantitation of cells infected by recombinant herpesvirus (pseudorabies virus) using a fluorescence-based .beta.-galactosidase assay and flow cytometry," J. Virol. Meth. 44:33-108 (1993).

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L11: Entry 5 of 7 File: USPT Jan 12, 1999

DOCUMENT-IDENTIFIER: US 5858682 A
TITLE: E2A/pbx1 fusion protein specific monoclonal antibodies

Detailed Description Paragraph Right (17):
A diagnostic system in kit form of the present invention includes, in an amount sufficient for at least one assay, a polypeptide, polypeptide admixture, antibody composition or monoclonal antibody composition of the present invention, as a packaged reagent. Instructions for use of the packaged reagent are also typically included.

Detailed Description Paragraph Right (19):
"Instructions for use" typically include a tangible expression describing the reagent concentration or at least one assay method parameter such as the relative amounts of reagent and sample to be mixed, maintenance time periods for reagent/sample admixtures, temperature, buffer conditions and the like.

Detailed Description Paragraph Right (26):
The reagent species, labeled specific binding agent or amplifying reagent of any diagnostic system described herein can be provided in solution, as a liquid dispersion or as a substantially dry powder, e.g., in lyophilized form. Where the indicating means is an enzyme, the enzyme's substrate can also be provided in a separate package of a system. A solid support such as the before-described microtiter plate and one or more buffers can also be included as separately packaged elements in this diagnostic assay system.

Detailed Description Paragraph Right (37):
The cells were attached onto precoated slides (BioRad, Munchen, Germany), fixed with 2% formaldehyde (in PBS) for 20 min., washed once with PBS and permeabilized for 30 min. at room temperature with 0.1% saponin (in PBS) containing 1% hydrogen peroxide. In order to block non-specific staining the cells were then incubated in 1% BSA containing 0.1% saponin (in PBS) for 10 min. before incubating them overnight at 4.degree. C. with purified antibody. The excess of unbound primary antibody was washed off and the cells then incubated for 30 min. with biotinylated donkey anti-mouse Ig (Jackson Immunoresearch Labs). After washing off excess unbound secondary antibody the cells were then incubated with horse-radish peroxidase (IWO) conjugated streptavidin reagent (Dako). The excess of unbound tertiary reagent was then removed and the cells were incubated in 0.1% 3,3' diaminobenzidine (DAB) solution containing 0.015% hydrogen peroxide and nickel chloride for 10 min. The cells were then washed extensively with water, counterstained with eosin, dehydrated in graded alcohol, cleared with xylene, and mounted with a coverslip for staining evaluation.

Detailed Description Paragraph Right (38):
Positive-reacting mAbs are used for mixing experiments to assess the sensitivity and lower limit of detection. These should be useful for post-chemotherapy monitoring of minimal residual disease and early relapse detection. The simultaneous use of additional markers to surface epitopes commonly expressed in pre-B ALL including CD10 and CD19 in two-color and three-color combinations enhance the level of sensitivity of assays utilizing an E2A/pbx1 fusion mAb. Finally, the correlation of reactivity with t(1;19)-positive ALL can be assessed cytogenetically to aid in the further identification and charactertization of the disease state.

Detailed Description Paragraph Right (39):

Cells were washed once with PBS containing 1% fetal calf serum and fixed with 4% paraformaldehyde for 1 hr at room temperature. The fixed cells were then washed twice with PBS, permeabalized with 100% methanol at -20.degree. C. for 5 min, and washed with PBS. Approximately 5.5.times.10.sup.6 cells were initially blocked with 1% BSA in PBS at room temperature for 20 min and then incubated with 1 .mu.g of E2A/pbx1 junction-specific monoclonal antibodies for 30 min, washed, stained with FITC-conjugated goat anti-mouse Ig (2.5 .mu.g/.mu.l) for 30 min, washed, and fixed with 2% of formaldehyde. For peptide 14 blocking experiments, the junction-specific antibodies were incubated with 0.1 .mu.g peptide 14 for 30 min at room temperature prior to their incubation with cells.

Detailed Description Paragraph Right (51):
To determine if the E2A/pbx1 fusion protein is shed by ALL cells, culture supernatants from t(1;19) translocation-positive and negative cell lines are passively absorbed on plates. The selected antibodies can be screened for their ability to detect the antigen if it is shown that the antigen is shed. Combinations of mAbs can be used for a capture and detection assay. In a second phase, the assay can be refined using serial serum samples from archived patient material.

Detailed Description Paragraph Right (70):
The intracellular staining of E2A/pbx1 using junction-specific antibodies with 697 cells was also demonstrated by flow cytometry. An anti-mouse IgG1 antibody was used as isotype control. Over 95% of 697 cells were stained by the junction-specific antibodies compared with <5% Namalwa which was similar to that of the isotype negative control antibody. The fluorescence of stained cells was intracellular, as there was no staining observed by junction-specific antibodies when cells were not fixed and permeabilized prior to the addition of antibodies. Furthermore, preincubation of 1 ng of peptide 14 with the monoclonal antibodies was capable of blocking 697 cell staining. These combined results indicate that the 697 cell staining was E2A/pbx1-specific. The significant difference of staining between E2A/pbx1 positive and negative cells implies that these antibodies can also be used for flow cytometric studies. At equal concentration, the G289-781 clone showed the strongest fluorescence staining in comparison with the other 2 tested clones.

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End of Result Set

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L11: Entry 7 of 7 File: USPT Jun 6, 1995

DOCUMENT-IDENTIFIER: US 5422277 A
TITLE: Cell fixative composition and method of staining cells without destroying the cell surface

Brief Summary Paragraph Right (2):
Since the outbreak of AIDS and other viral-related disease states there has been an increased need for new and better methods of studying the etiology and pathology of viruses, as well as therapeutic monitoring of treatment regimes for arresting or modifying in some way the effect these viruses have on cells, and ultimately the patient. For example, it is often desirable to assay the percentage of cells infected. A high virus burden usually means the disease is rampant, while a low virus burden might mean that the particular disease is in its early stages, or is responding to therapeutic treatment, and so on. Conventional assays for measuring virus burden have to date been merely extrapolative in nature. For example, cells may be cultured and titrated out for analysis. In these assays, it is common to employ polymerase chain reaction amplification techniques, in an attempt to quantitate copies of DNA (as the provirus form) or RNA present, the precept being, the more RNA, the more virus. However, results obtained with techniques of this sort can only indicate without distinction, a small quantity of infected cells having a high quantity of virus burden, or a larger quantity of cells with a low virus burden per cell. Another drawback is that a determination in accordance with this technique does not provide information as to whether or not the virus infection is replicative or abortive, since one does not know "how many" or "which" cells are infected. Hence, true virus burden assessment, as well as virus activity cannot be had with this technique. Furthermore, it is difficult, cumbersome, and costly to implement, all without giving results that are acceptable in sensitivity.

Brief Summary Paragraph Right (3):
Virus infection may also be monitored by monitoring the quantity of a viral component such as p24 (in the case of HIV-1) present in a patient's serum. However, this technique is grossly extrapolative and often given to false negatives. For example, a patient may demonstrate a short spike in p24 concentration at the beginning of infection, when the virus is replicating, but before that patient's antibody response to this virus. Within a period of 5 days to about 2-3 weeks, the patient will start to make antibodies to p24. These antibodies bind to the p24 in plasma and either remove it from circulation or block its detection in immunoassays. Accordingly, there is a very short window in which to detect the p24 antigen component, as the patient will test negative once he is making antibodies to the p24. It is not until the patient becomes so compromised that he can no longer produce antibodies to the p24 component, that the test begins to again indicate a positive result for the p24 antigen. Unfortunately, the patient prognosis is very grim at this point, as the disease has progressed past the stage of responding to any therapeutic treatment.

Brief Summary Paragraph Right (5):
In particular, there is a specific need for routine monitoring of virus load in HIV-infected individuals, preferably, through the use of a fixative that inactivates the virus and thus, increases the safety of handling samples containing this deadly virus. This information will be used by physicians to categorize HIV disease states, monitor and document progression, assess prognosis, and possibly to better tailor effective therapeutic regiments on an individual basis. Additionally, pharmaceutical companies and researchers require just such an assay for use in clinical trials, to

determine rapidly if a proposed therapeutic agent is both safe and effective at controlling virus load.

Brief Summary Paragraph Right (7):
The above-described technique may be used with a patient's cells, to analyze for the presence of viral antigens. Interfering antibodies present in such patient's blood sample are simply washed away with the serum prior to this flow cytometric analysis. However, virion particles, if present, are in the cellular cytoplasm, and sometimes the nucleus. In order to look at the viral antigens inside the cell, the cellular membrane must be permeated to allow antibodies against the virus to enter the cell. Using the prior art techniques of the past, all or a portion of the cellular surface would be stripped away to allow the large antibody to enter. Typically this is done through the use of agents such as methanol or other alcohols which tend to extract lipids and precipitate proteins. Such agents basically turn the cell into a bead of protein, and in this manner provide access to the proteins that may be present. However, in so doing, the cell's surface characteristics are destroyed.

Drawing Description Paragraph Right (3):
FIG. 3 is a cytogram obtained with the use of a FACScan, demonstrating the results of a double antibody staining assay.

Detailed Description Paragraph Right (15):
Preferred for use herein as this third or fourth component, are one or a combination of zwitterionic or non-ionic surfactants such as sodium cholate, deoxycholates, CHAPS, saponin, and polymers of ethylene oxide, such as ethoxylated alcohols, ethoxylated alkyl phenols, ethoxylated amines and amides, polyoxyethylene sorbitans of the "Tween.TM. series, such as monolaurate (Tween 20), monopalmitate (Tween 40), monooleate (Tween 80), and polyoxethylene-23-lauryl ether (Brij.TM. 35), polyoxyethylene ether W-1 (Polyox), and the like. One skilled in the art will understand that a good description of compounds belonging to the foregoing classifications, and vendors from whom such compounds may be commercially obtained may be found in "Chemical Classification, Emulsifiers and Detergents", McCutcheon's, Emulsifiers and Detergents, 1986, North American and International Editions, McCutcheon Division, MC Publishing Co., Glen Rock, N.J., U.S.A. and Judith Neugebauer, A Guide to the Properties and Uses of Detergents in Biology and Biochemistry, Calbiochem.RTM., Hoechst Celanese Corp., 1987. Preferred among these are the polyoxyethylene sorbitans of the "Tween.TM." series available from Sigma Chemicals, or the Triton.TM. series, available from Rohm and Haas of Philadelphia, Pa., and especially Triton.TM. X-705, Triton.TM. X-100, and Triton.TM. X-114, available commercially from many vendors. Preferred concentrations of these range from about 0.001 to about 0.2% (w/v), with about 0.05 to about 0.1% (w/v) particular preferred.

Detailed Description Paragraph Right (22):
The use of light scatter is illustrated in FIGS. 1 and 2, which are three-dimensional representations of the number of cells having light scattering properties as measured on a FACScan flow cytometer available from Becton Dickenson Corporation. Forward light scatter was plotted on the abscissa (X-axis) 10, versus SSC on the ordinate (Y-axis) 12. The number of cells is represented on the Z-axis, 14. The intensity of FSC and SSC for each cell was measured and given a relative value on a scale of 1 to 1000. FIG. 1 shows the results using live unfixed cells, with 100 representing the lymphocyte cluster, 102 representing the monocyte cluster, and 104 representing the granulocyte cluster respectively. FIG. 2 shows the results using cells after having been fixed with the fixative composition of the present invention. Although fixed and made permeable, it was evident that cell clusters remained well defined. Because light scatter depends on factors such as cell size, cytoplasmic complexity and the cell's index of refraction, it was not surprising to find that the light scatter of fixed cells had varied a little from unfixed cells. However, it was surprising to find that after fixation, not only could the three populations of cells be resolved by light scatter alone, but that the separation of lymphocytes, monocytes and granulocytes was improved.

Detailed Description Paragraph Right (25):
A more complicated assay would employ a second antibody added simultaneously. In this case, the second antibody would have a different specificity from the first,

and be coupled with a dye that emits light of a different color. Typically, phycoerythrin (PE) is used for this purpose, because it too absorbs blue light, but fluoresces yellow. In this way, one can determine whether a cell reacts with either the FITC labeled antibody, the PE labeled antibody or both. For example, the antigen known as DR is not expressed on T-cells unless the cells have been activated. In contrast, the DR antigen is expressed on most B-cells. If white blood cells are reacted with anti-CD3-FITC and anti-DR-PE antibodies, all four possible combinations of reactivity would be expected. Some lymphocytes would not react with either antibody and would only scatter light. The non-activated T-cells would react with anti-CD3 antibody and emit only green light. B-cells would react with anti-DR antibody and emit only yellow light. However, activated T-cells would react with both antibodies and emit both green and yellow light. Such a situation is illustrated in FIG. 3, which is a cytogram obtained with the use of the FACScan. Here white cells have been reacted with anti-CD3-FITC and anti-DR-PE and the fluorescence of the lymphocytes, selected as lymphocytes on the basis of their light scatter, has been plotted with each cell's green fluorescence intensity on the abscissa 10, and its yellow fluorescence intensity on the ordinate 12. Unreactive cells cluster at the origin 16. B-cells are displaced vertically, in a cluster 18 distinct from unreactive cells but directly above it. Unactivated T-cells are displaced along the abscissa in a cluster 20 distinct from unreactive cells but directly along side of them. Finally, activated T-cells 22 emit just as much green light as unactivated T-cells because they express the same amount of CD3, but they are also displaced vertically because they co-express the DR antigen, and having reacted with the anti-DR-PE antibody, emit yellow light.

Detailed Description Paragraph Right (26):
The situation may be made even more complex by the inclusion of a third antibody or a DNA/RNA stain that absorbs blue light and emits red light. This arrangement of reagents is not discussed in detail because it does not illustrate any principles of flow cytometry not already covered by the previous discussion of one and two color reagent analysis; however, it does serve to illustrate how complex and sophisticated flow cytometric analysis of cells may become. By way of example, if the third reagent is a DNA stain, then the amount of DNA in a cell may be measured. The amount of DNA is dependent upon whether the cell is at rest, synthesizing DNA in preparation for cell division, or is about to divide. Quantitating the amount of DNA allows the user to selectively examine cells in various stages of the cell cycle. It then becomes possible to determine whether certain antigens are always expressed by a cell, or only present during restricted portions of the cell cycle. Such data is analyzed essentially as done for one and two color assays, but with greater appreciation for the complexity of simultaneous reactions being measured.

Detailed Description Paragraph Right (39):
Cells were washed once in PBS, then resuspended in 4 mLs of PBS (43.times.10.sup.7 cells/mL). The cells were separated into 4 1 mL aliquots. The first tube received 1 mL of PBS and tubes 2 through 4 received 1 mL of 1% paraformaldehyde. All tubes were incubated for 30 min at room temperature. All tubes were washed twice in PBS. Tubes 1 and 2 were put in ice until needed. Cells in tubes 3 and 4 were resuspended in 1 mL of PBS. To tube 3 was added 1 mL of 0.5% (wt/v) Nonidet P40 (NP40; BDH Limited), and it was incubated 30 minutes at room temperature. To tube 4 was added 6.6 mLs of methanol (-70.degree. C.) and it was incubated 30 minutes at 0.degree. C.

Detailed Description Paragraph Right (47):
In order to visualize intracellular antigens, it is not enough to simply fix cells. Cells must also be made permeable to molecules as large as antibodies. The methods most frequently employed to permeabilize cells are methanol or detergent treatment of fixed cells (reviewed in Jacobberger, J. W., (1989) Cell cycle expression of nuclear proteins. In A. Yen (ed.), Flow cytometry: advanced research and clinical applications. CRC Press, Inc. Boca Raton Fla.). Methanol further fixes proteins, randomizes their conformation and extracts membrane lipids. Detergent treatment extracts membrane lipids, creating holes where antibodies may pass freely in and out of the cell.

Detailed Description Paragraph Right (48):
Immunostaining and light scatter properties of cells fixed in 1% PF versus cells fixed in1% PF followed by methanol permabilization was measured. The ability to

discriminate lymphocytes from monocytes on the basis of their FSC is diminished by methanol treatment. In addition, methanol treated cells had increased nonspecific binding of control-FITC antibody. The most important finding, however, was that methanol treated cells no longer reacted with OKT3, OKT4, and still did not react with OKT11.

Detailed Description Paragraph Right (54):
To determine whether fixation had made cells permeable to antibody while retaining their cytoplasmic antigens, live and fixed cells were reacted with mouse monoclonal antibodies specific for the cytoplasmic proteins gelsolin and vimentin. Anti-gelsolin (clone No. GS-2C4, Sigma Chemical Co.) and anti-vimentin (clone No. V9, Sigma Chemical Co.) were not conjugated to fluorescent dyes. Therefore, binding of these antibodies could not be determined "directly." Instead, binding of these antibodies to cells was determined "indirectly" by reacting treated cells with goat anti-mouse IgG-FITC. Immunostaining of cytoplasmic antigens was done by adding 10 uL of Control IgG2a mouse antibody (10 ug/mL), or 10 uL of anti-gelsolin (diluted 1/100 in block solution) or 10 ul of anti-vimentin (diluted 1/60 in block solution) to 100 ul of fixed or live cell suspension. Cells and antibody were incubated for one hour as described above, then washed three times in PBS/S using 2 mLs per wash. After the last wash, the supernatant fluid was removed by aspiration, and the cells resuspended in 100 uL of block solution. Each suspension then received 200 uL of goat anti-mouse IgG-FITC conjugate (F(ab').sub.2, Sigma Chemical Co.) diluted 1/75 in block solution. Cells were again incubated for 60 minutes, then washed three times in PBS/S, using 2 mL per wash. After the last wash, cells were resuspended in 0.5 mL of PBS/S and analyzed on a FACScan flow cytometer.

Detailed Description Paragraph Right (57):
OK-Control-FITC is a mouse IgG2a antibody that does not react with any known cellular antigens. Therefore, any green fluorescence emitted by cells following treatment with this antibody is due to nonspecific binding of antibody to cells. The nonspecific binding by live and fixed cells was determined. When reacted with OK-Control, 100% of live cells had a fluorescence intensity less than 3.92 (on a scale of 1 to 10.sup.4) with a mean intensity for live cells of 1.20. Nonspecific binding of antibody to fixed cells was only slightly higher than that of live cells. 99.5% of fixed cells had fluorescence less than 13.82, with a mean fluorescence intensity of 3.99. The data therefore demonstrated that cells fixed and stained by the present reagent and methods do not bind significant quantities of antibodies nonspecifically. Staining of the cell surface molecule CD3 by OKT3-FITC antibody was measured. 77.4% of live lymphocytes were positive for CD3, compared to 77.9% of the fixed lymphocytes. The mean fluorescence intensity for CD3 positive live cells was 188.83. The mean fluorescence intensity of CD3 positive fixed cells was 144.68; 23% lower than the intensity of live cells. Although the intensity of staining was modestly lower, the reagent and methods as described herein did not destroy the ability of OKT3 to bind CD3 on fixed cells; and enough OKT3 was bound by fixed cells that CD3 positive lymphocytes could be separated unequivocally from CD3 negative lymphocytes, based on their fluorescence intensity. This result is in marked contrast to cells fixed and made permeable by paraformaldehyde and methanol treatment as performed in the art, where the ability of OKT3 to bind CD3 had been destroyed completely (see Comparative Experiment 2 above).

Detailed Description Paragraph Right (58):
Staining of the cell surface molecule CD4 by OKT4-FITC antibody was also performed. 55.1% of live lymphocytes were positive for CD4, compared to 52.7% of the fixed lymphocytes. The mean fluorescence intensity for CD4 positive live cells was 69.57. The mean fluorescence intensity of CD4 positive fixed cells was 63.74; 8% lower than the intensity of live cells. Although the intensity of staining was modestly lower, the present invention's reagent and methods did not destroy the ability of OKT4 to bind CD4 on fixed cells; and enough OKT4 was bound by fixed cells that CD4 positive lymphocytes could be separated unequivocally from CD4 negative lymphocytes based on their fluorescence intensity. This result is in marked contrast to cells fixed and made permeable by paraformaldehyde and methanol treatment, where the ability of OKT4 to bind CD4 had been destroyed completely (see Comparative Experiment 2 above).

Detailed Description Paragraph Right (59):
Staining of the cell surface molecule CD2 by OKT11-FITC antibody was also performed.

85.3% of live lymphocytes were positive for CD2, compared to 84.3% of the fixed lymphocytes. The mean fluorescence intensity for CD2 positive live cells was 49.95. The mean fluorescence intensity of CD2 positive fixed cells was 41.00; 18% lower than the intensity of live cells. Although the intensity of staining was modestly lower, the present invention's reagent and methods did not destroy the ability of OKT11 to bind CD2 on fixed cells; and enough OKT11 was bound by fixed cells that CD2 positive lymphocytes could be separated unequivocally from CD2 negative lymphocytes based on their fluorescence intensity. This result is in marked contrast to cells fixed and made permeable by paraformaldehyde and methanol treatment, where the ability of OKT11 to bind CD2 had been destroyed completely (see Comparative Experiment 2 above). In addition, when lymphocytes were treated with paraformaldehyde followed by NP40 solubilization of membranes, some CD3 and CD4 staining was observed, but no staining of CD2 by OKT11 was seen.

Detailed Description Paragraph Right (72):
SEDA provides the researcher with the ability to study many more variables simultaneously by reducing the number of combinations required within the experiment. In the example of the experiment having 3 variables, the 3 variables may be called X, Y and Z. The range of values from low to high for X, Y and Z within the experiment may be thought of as defining the dimensions of a cube; with X representing the width, Y the height and Z the depth. A full three level factorial design would require a test at each corner of the cube and all midpoints (27 experiments in all). The Box-Behnken design requires testing only the combinations that represent the mid-point of each edge of the cube, and triplicate determinations of the combination that represents the center of the cube. This reduces the number of combinations from 27 to 15. The advantage of the Box-Behnken design becomes dramatic as the numbers of variables increase. A Box-Behnken design requires only 27 combinations for 4 variable and 46 for 5 variables; compared to 81 and 243 respectively for a three level factorial design. In addition, after the data has been collected and the computer selects mathematical models that correspond to the observed experimental results, SEDA can be used to predict the performance of the assay at any point on the surface or within the volume of the cube. Experiments were designed using SEDA. A file was created where the variables to be studied (eg. formaldehyde, DNBS, DMSO and detergent concentrations) and the performance to be measured ie., staining of cytoplasmic or surface antigen) were entered into the computer. After specifying the upper and lower limit for each variable, the software determined the concentration of each variable for each "run" within the experiment (see Table E3-1). The runs were then put in random order. After the laboratory portion of the experiment was completed, the measured performances corresponding to each run were entered into the computer. The computer determined those variables that had the greatest effect on performance, those that had no effect, and those that interacted synergistically. Regression lines were fitted to the experimental data using linear, interactive and quadratic equations. The mathematical model giving the best fit to the data was chosen and carefully checked for how well it predicted the experimental results.

Detailed Description Paragraph Right (73):
The computer model could be used to predict assay performance at any concentration or any combination of concentrations within the range of each variable tested. With this capability, countless combinations of reagent concentrations were tested by computer simulation. The ability of these simulated fixative formulations to allow staining of cytoplasmic antigens, or in some cases the degree of harm done to cell surface antigen staining, was predicted. Furthermore, minimum performance criteria were set and an algorithm in the software used to calculate the optimum combination of reagent concentrations to achieve any given desired performance. Once optimized, the computer was instructed to hold all variables at their optimal level except one. One variable was then varied over its entire range and its effect on performance plotted.

Detailed Description Paragraph Right (80):
Different detergents have been used in the preparation of the fixative composition of the present invention. The detergents were not only different in composition, but represent distinct classes of compounds. The fixative of the present invention was prepared as has been previously described, except in this experiment, the detergent and the detergent concentration were varied. The detergents used were

polyoxyethylene ether W-1 (Polyox), polyoxyethylenesorbitans monolaurate (Tween 20), monopalmitate (Tween 40), monooleate (Tween 80) and polyoxyethylene 23 lauryl ether (Brij 35). Other detergents that work well include Nonidet P-40, Triton X-100, sodium deoxycholate and saponin.

Detailed Description Paragraph Right (87):
Viruses grow within cells. The nucleic acids and proteins that constitute the viral particle are produced by the infected cell and accumulate in the cell. It should be possible to detect the presence of viral proteins in infected cells if the virus is transcriptionally active. The human immunodeficiency virus (HIV) produces many proteins. Some of the proteins regulate viral gene expression and some are structural proteins that make up the core or the envelope of the virus. The protein p24 is a structural protein that HIV infected cells produce in excess. The ability to detect replicating virus in cells may have clinical significance in detecting and monitoring the disease, acquired immunodeficiency syndrome (AIDS), caused by HIV. The virus load in HIV-infected individuals is related to disease progression and prognosis. In the past, and as previously described in the Background of the Invention, virus load has been monitored through the use of culture, polymerase chain reaction (PCR) or the p24 immunoassay. HIV culture and PCR are costly specialized tests, not amenable to most clinical laboratory environments. The p24 assay is often negative in HIV infected individuals, because immune complexes between p24 and the patient's own antibody prevent capture of p24 in commercial assay kits.

Detailed Description Paragraph Right (88):
To determine if the reagents and methods of the present invention could be used to detect viral p24 within infected cells, HIV infected tissue culture cells and peripheral blood leukocytes from HIV infected individuals were examined. The human tissue culture cell line H9 is capable of supporting the growth of HIV. Uninfected H9 cells were obtained from the National Institutes of Health's AIDS Research and Reference Reagent Program. H9 cells, persistently infected with HIV, were obtained from the American Type Culture Collection. Uninfected and persistently infected H9 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum. Cultures were passed at least once per week. Blood from three individuals known to be infected with HIV was obtained and stored at room temperature in EDTA for less than 24 hours before being fixed. After fixation, the cells were frozen at -20.degree. C. until thawed for an HIV p24 assay.

Detailed Description Paragraph Right (93):
FIGS. 6a, 6b, 6c and 6d show the results when blood from HIV infected individuals and an uninfected control individual was tested. Side scatter was plotted on the X-axis and the fluorescence intensity of anti-p24 binding was plotted on the Y axis. Side scatter alone was not able to resolve monocytes from granulocytes in this experiment. This was most likely due to the cells having been frozen and thawed before being assayed. When patient cells had not been frozen and thawed prior to being used, light scatter could differentiate fixed lymphocytes, monocytes and granulocytes of HIV infected and AIDS patients as well as it could for uninfected individuals. FIG. 6a shows the result for a control HIV uninfected hospital patient. There is no evidence of anti-p24 antibody binding to cells in either the lymphocyte cluster on the left or in the monocyte-granulocyte cluster on the right. FIG. 6b shows the result from an individual classified as CDC stage III disease and taking the drug Zidovudine (AZT). The individual was asymptomatic at the time of blood draw. Note the distribution of the lymphocyte cluster is skewed upward on the Y-axis compared to the control cells. A number of lymphocytes bound a low level of anti-p24 in this patient. In addition, a cluster of a small number of brightly staining monocytes were clearly evident in this patient. FIG. 6c shows the results of an individual whose disease had progressed to CDC stage IV. This individual was suffering from oral candidiasis at the time the blood was drawn. In this patient the lymphocyte cluster was completely negative for anti-p24 binding. Granulocytes too seemed free of viral proteins but there was a striking involvement of the patient's monocytes. Finally, FIG. 6d presents the results of a patient with full blown AIDS. This individual had developed a peripheral neuropathy as a consequence of drug treatment and had to be removed from anti-retroviral chemotherapy. It was evident that numerous lymphocytes, monocytes and granulocytes bound anti-p24 antibody in this patient. Many cells bound so much anti-p24, their fluorescence was off scale.

Detailed Description Paragraph Right (103):
The HTLV-III-B strain of HIV-1 was used as the target virus. MT-4 cells were used as the host cell for growing the virus. MT-4 cells were infected with HIV-1 virus and incubated for 48 hours. Approximately 8.times.10.sup.6 cells were pelleted, then resuspended in 0.8 mL of cell free HIV-1 virus. 0.4 mLs of this mixture was then diluted with 0.4 mL of fetal bovine serum to make the mixture 50% serum. A 0.1 mL sample was removed to test the total viral burden; this sample was labeled "Viral Load". The rest of the virus/cell suspension (0.7 mL) was mixed with 0.7 mL of fixative. A 0.1 mL sample was immediately removed and diluted 1:300 in RPMI 1640+10% FBS. This represented the T=0 sample. The rest of the suspension was incubated 30 minutes at room temperature. At 30 minutes, a 0.1 mL sample was again taken and diluted 1:300 in RPMI 1640+10% FBS, this represented the T=30 sample. Both the T=0 and T=30 samples were serially diluted and 0.1 mL samples inoculated into dishes containing 1.0 mL of uninfected MT-4 cells. The MT-4 cell cultures were not washed free of the inoculum, but cultures were fed twice per week by removing 1.0 mL of medium and replacing it with a fresh 1.0 mL. Cultures were examined on days 7, 14 and 28 post inoculation for the presence of cytopathic effect (CPE). Cytopathic effect is a morphologic change to an infected cell that occurs as a result of viral growth. In addition, supernatant fluids from 7, 14 and 28 day cultures were collected and assayed for the presence of HIV-1 specific viral p24 protein.

Detailed Description Paragraph Center (7):
Methanol Permeation